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Original Article

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Measurements of echocardiographic indices and biomarkers of kidney injury in dogs with chronic kidney disease

M.J. Hezzell [a,b,*], J.D. Foster [a,c], M.A. Oyama [a], J. Buch [d], G. Farace [d], J.J. Quinn [d], M. Yerramilli [d]

[a] *Department of Clinical Studies - Philadelphia, School of Veterinary Medicine, University of Pennsylvania, Philadelphia PA 19104 USA*

[b] *Bristol Veterinary School, University of Bristol, Langford House, Langford, Bristol, BS40 5DU, UK*

[c] *Friendship Hospital for Animals, 4105 Brandywine Street NW, Washington, DC 20016, USA.*

[d] *IDEXX Laboratories Inc, One IDEXX Drive, Westbrook ME 04092 USA*

* Corresponding author. Tel.: +44 117 331 9306

E-mail address: mh16511@bristol.ac.uk (M.J. Hezzell).

Abstract

Pathophysiological cardiac and renal interactions are termed cardiovascular-renal disorder (CvRD). Cardiovascular disease/dysfunction secondary to kidney disease ($CvRD_K$), is a leading cause of death in human chronic kidney disease (CKD) patients. The presence and clinical impact of $CvRD_K$ in dogs with CKD is unknown. We hypothesized that echocardiographic measurements, and cardiac and renal biomarkers, will be altered in dogs with CKD and associated with survival. Eleven dogs with CKD (n=6 IRIS stage 2, n=5 IRIS stage 3) and without primary cardiac disease, plus 12 healthy age-matched control dogs, were recruited to this prospective observational study. Dogs underwent standard echocardiography, glomerular filtration rate (GFR) estimation by iohexol clearance, and measurement of plasma cardiac troponin I and N-terminal pro-B-type natriuretic peptide (NT-proBNP), plasma and urinary cystatin B, and urinary clusterin and neutrophil gelatinase-associated lipocalin (NGAL). Values were compared between groups, and their association with all-cause mortality explored.

Dogs with CKD had significantly lower GFR and higher NT-proBNP, urinary cystatin B, clusterin, and NGAL, compared to controls ($P<0.05$). Echocardiographic measurements were similar between dogs with CKD and controls. Median follow-up time was 666 days, during which six dogs with CKD died. Risk of death was associated with increasing age, serum total protein, and normalized left ventricular posterior wall thickness (LVPWDN) and decreasing bodyweight and packed cell volume. Although baseline differences in echocardiographic measurements were not evident between dogs with moderate CKD and controls, the presence of $CvRD_K$ was suggested by the association between LVPWDN and survival.

Keywords: Biomarker; Canine; Cardiorenal syndrome; Cardiovascular physiology.

Introduction

By virtue of their roles in fluid balance, tissue perfusion, and blood pressure, the physiology of the heart and kidney are inextricably intertwined (Kingma et al., 2017). As a result, disease or dysfunction of one system may lead to disease or dysfunction of the other, which has been described as cardiorenal syndrome in humans (Ronco et al., 2008; Rangaswami et al., 2019). In veterinary medicine, a similar syndrome, termed cardiovascular-renal disorders (CvRD), has been proposed (Pouchelon et al., 2015). CvRD describes three different circumstances as follows: kidney disease/dysfunction that arises secondary to heart disease ($CvRD_H$), cardiovascular disease/dysfunction that arises secondary to kidney disease ($CvRD_K$) and disease/dysfunction of both cardiovascular and kidney systems due to either concurrent primary heart and kidney disease or other pathophysiological mechanisms affecting both systems (e.g. systemic inflammation, drug or other intoxication, etc.) ($CvRD_O$) (Pouchelon et al., 2015).

Development of cardiovascular disease as a result of chronic kidney disease (CKD) is a well-characterized and important disease mechanism in human patients (Ronco and Di Lullo, 2014). Patients with CKD are at 3-30 times higher risk for development of heart disease, and the majority of patients with advanced CKD die from congestive heart failure, myocardial infarction or arrhythmias as opposed to kidney failure (Muntner et al., 2002; Granata et al., 2016). Diagnosis of cardiorenal syndrome is challenging, as the mere coexistence of both heart and kidney disease is insufficient evidence of this syndrome. With regards to cardiovascular disease that arises secondary to kidney disease, kidney disease must causally trigger the development of heart disease. Potential pathophysiological mechanisms include acute

neurohormonal activation, inflammation, oxidative stress, and endothelial dysfunction (Herzog et al., 2011; Alhaj et al., 2013), all of which can result in left ventricular systolic or diastolic dysfunction, volume overload, pressure overload, and myocardial remodeling. Important aspects to better understanding cardiorenal syndrome in patients with CKD include characterization of "normal" heart function at baseline, detecting longitudinal changes in function, and identifying patient and laboratory characteristics that influence outcome (Clementi et al., 2013; Pinheiro da Silva and Vaz da Silva, 2016). Interest in CvRD in veterinary medicine is a relatively recent phenomenon, and CvRD is incompletely described and understood (Pouchelon et al., 2015; Keller et al., 2016; Martinelli et al., 2016; Jung et al., 2018). To the authors' knowledge, CvRD and its association with survival in dogs with CKD has not been specifically investigated. The primary objective of this study was to describe baseline cardiac measurements and kidney function using echocardiography, biochemical parameters and biomarkers in a cohort of dogs with moderate CKD (International Renal Interest Society (IRIS) disease stages 2 and 3) compared to age- and weight-matched healthy dogs, and to explore the effect of these parameters on long term outcome. The secondary objective was to provide pilot data that could be used to perform a sample size calculation for a larger, more definitive future study; for this reason no *a priori* sample size calculation was performed.

Materials and Methods

The study was approved by the University of Pennsylvania animal use and care committee (Approval number, IACUC 805002; Approval date, 21 July, 2014), and informed owner consent was obtained. Dogs presenting to the Ryan Veterinary Hospital of the University of Pennsylvania for CKD were prospectively recruited. The

diagnosis of CKD was made on the basis of at least two episodes, over at least 3 months, of minimally-concentrated urine (urine specific gravity < 1.030) with stable azotemia in the absence of other causes of polyuria or polydipsia. Additional factors considered included identification of renal proteinuria or ultrasonographic changes consistent with CKD (e.g., loss of corticomedullary distinction, irregular contours, and decreased renal size). Based on IRIS staging guidelines, dogs were assigned to either IRIS stage 2 (serum creatinine concentration 1.4 – 2.0 mg/dL) or stage 3 (serum creatinine concentration 2.1 – 5.0 mg/dL) CKD.[1] The exclusion criteria included non-azotemic and advanced CKD (i.e. IRIS stages 1 and 4), hypothyroidism, bodyweight <10kg, detection of a heart murmur on auscultation, echocardiographic evidence of primary heart disease, specifically degenerative mitral valve disease or idiopathic dilated cardiomyopathy (Dukes-McEwan et al., 2003), presence of an active urine sediment, or recent glucocorticoid or diuretic therapy. Therapy with amlodipine or an angiotensin converting enzyme inhibitor for control of systemic hypertension was permitted. A second cohort of healthy age- and weight-matched controls was recruited concurrently, primarily from students and hospital employees.

A medical history was taken, and physical examination, blood sampling, blood pressure measurement, measurement of glomerular filtration rate (GFR) and echocardiography were performed. Systolic blood pressure was measured in a quiet area following a period of acclimation for 5-10 min (Acierno et al., 2018). Dogs were placed in lateral recumbency, and systolic blood pressure measured using Doppler sphygmomanometry using the uppermost thoracic limb and an inflatable cuff closest

[1] See: IRIS International Renal Society. http://www.iris-kidney.com/pdf/003-5559.001-iris-website-staging-of-ckd-pdf_220116-final.pdf#page=6 (Accessed 5 December 2019)

to 40% of the antebrachial circumference (Ultrasonic Doppler Flow Detector, Parks Medical). The first measurement was discarded and the mean of 5-7 consecutive consistent measurements recorded. GFR was estimated by a reference laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI), using the plasma clearance of iohexol method, as previously described (Heiene et al., 2010). Blood samples were obtained via jugular or cephalic venipuncture at baseline and 2, 3 and 4 h post-iohexol administration, with sample volumes of 5.5mL, 3 mL, 3mL and 3mL, respectively, collected at each time point. At baseline, blood samples were divided between plain and K-EDTA and lithium heparin treated tubes. At the 2, 3 and 4 h post-iohexol time points all blood was collected into plain tubes. Serum biochemistry and plasma cTnI were performed by the University of Pennsylvania clinical laboratory using samples collected at baseline. K-EDTA samples were separated by centrifugation at 1000g for 15 min and an aliquot of the resultant plasma transported at ambient temperature to a reference laboratory (IDEXX Laboratories) for analysis of NT-proBNP concentration using a second generation ELISA assay that has been previously validated in dogs (Cardiopet Canine proBNP, IDEXX Laboratories; Cahill et al., 2015). A second aliquot of K-EDTA plasma was stored at -80 °C for subsequent batched analysis of plasma symmetric dimethylarginine (SDMA) and cystatin B at a reference laboratory (IDEXX Laboratories).

Urine samples were collected by cystocentesis. Urine specific gravity (USG) was measured using refractometry immediately following sample collection. Each sample was divided into two aliquots. One aliquot underwent routine urinalysis and measurement of the urinary protein to creatinine ratio (UPC), performed by the University of Pennsylvania clinical laboratory. The second aliquot underwent

centrifugation at 1000g for 15 min. The supernatant was stored at -80 °C for subsequent batched analysis of urinary cystatin B, neutrophil gelatinase-associated lipocalin (NGAL), and clusterin at a reference laboratory (IDEXX Laboratories). All blood and urine sample processing was completed within 2 h of collection. All biomarker analyses were performed in duplicate and the mean of the two measurements reported.

Kidney biomarker assays for plasma SDMA, plasma and urinary cystatin B, and urinary NGAL and clusterin were performed as previously described (Nabity et al., 2015; Yerramilli et al., 2016). Echocardiographic examinations were performed either by a board-certified cardiologist or a cardiology resident-in-training under the direct supervision of a board-certified cardiologist. Dogs were placed in right, then left, lateral recumbency on an ultrasound examination table. The echocardiographic examination was performed using an ultrasound unit equipped with 3-8 MHz and 1-5 MHz phased array transducers and ECG monitoring (iE33, Phillips). Standard imaging planes were digitally stored.

The left atrial to aortic root ratio (LA:Ao) was measured from the right parasternal short axis view (Haggstrom et al., 1994; Hansson et al., 2002). Left ventricular internal dimension in systole (LVIDs), left ventricular internal dimension in diastole (LVIDd), interventricular septal thickness in diastole (IVSd), and left ventricular posterior wall thickness in diastole (LVPWd) were measured from 2D right parasternal short axis views. LVIDs was normalized for bodyweight (LVIDSN) using the following formula:

$$\text{LVIDs} / [\text{bodyweight (kg)}]^{0.315}$$

LVIDd was normalized for bodyweight (LVIDDN) using the following formula:

LVIDd/[bodyweight (kg)] $^{0.294}$

LVPWd was normalized for bodyweight (LVPWDN) using the following formula:

LVPWd/[bodyweight (kg)] $^{0.232}$

IVSd was normalized for bodyweight (IVSDN) using the following formula:

IVSd/[bodyweight (kg)] $^{0.241}$ (Cornell et al., 2004)

Tissue Doppler imaging profiles were obtained of the lateral mitral annulus, as previously described (Dickson et al., 2017).

Statistical methods

Statistical analysis was performed using commercially available software (SPSS 23, IBM; GraphPad Prism 7.00, GraphPad Software). *P* values < 0.05 were considered statistically significant. Due to the small sample size, median (range) values were reported for continuous variables. Comparisons of continuous variables between groups were performed using Kruskal-Wallis tests with Dunn's post hoc tests for multiple comparisons. Correlation between variables was assessed using Spearman correlation coefficients. Survival time of dogs with CKD was calculated from date of enrolment to death, euthanasia, or end of study follow up (November 2017). Cause of death of dogs was determined by review of medical records and

telephone interview. Dogs were censored if they were still alive, or if they had been lost to follow-up. Univariate Cox proportional hazards models were constructed to investigate whether selected variables were associated with survival.

Results

The study was performed between October 2014 and November 2017. Eleven dogs with CKD, including five in IRIS stage 2 (four females and one male), and six in IRIS stage 3 (four females and two males), were recruited between October 2014 and May 2016 and included in the study. Cross-breeds were most frequently represented (n = 3), followed by two each of old English sheep dog and golden retriever, and one each of four other breeds. Twelve healthy control dogs, including seven males and five females, were recruited between November 2014 and July 2016. Cross-breeds were most frequently represented (n = 4), followed by one each of eight other breeds. A summary of the physical examination findings and routine biochemical testing is shown in Table 1. All dogs underwent indirect ophthalmic examination by a board-certified internist as part of their complete physical examination. No retinal lesions consistent with hypertensive retinopathy were observed. Eleven dogs had systolic blood pressure measurements >160 mmHg, including 3/12 dogs in the control group, 4/5 dogs in the IRIS stage 2 group and 4/6 dogs in the IRIS stage 3 group. Three dogs in the IRIS stage 3 group were receiving medications. Two dogs were receiving an ACE inhibitor (systolic blood pressures were 130 and 146 mmHg; both dogs had previously been documented to be proteinuric), and one dog was receiving amlodipine (systolic blood pressure was 180 mmHg). No dog in IRIS stage 2 had previously been diagnosed with systemic hypertension and none were receiving anti-hypertensive medications.

Summary statistics for GFR, biomarker, and echocardiographic data from the three groups are shown in Table 2. Plasma SDMA and cystatin B, and urinary cystatin B, NGAL, and clusterin were measured in samples that had been stored at -80°C for 2-27 months. GFR was significantly lower in dogs with IRIS stage 3 compared to healthy controls ($P < 0.001$) but did not differ between controls and IRIS stage 2, or IRIS stages 2 and 3 ($P = 0.050$ and 0.482, respectively). An overall difference between groups was detected for SDMA ($P < 0.0001$), plasma NT-proBNP ($P = 0.001$), urinary cystatin B/ creatinine ratio ($P = 0.005$), urinary clusterin/ creatinine ratio ($P = 0.019$) and urinary NGAL/ creatinine ratio ($P < 0.001$; Table 2). NT-proBNP was moderately correlated with GFR ($r_s = -0.610$; $P = 0.003$), and serum creatinine ($r_s = 0.588$; $P = 0.004$).

Vital status of dogs with CKD was determined in November 2017. Median follow-up time was 666 days (range, 20-1085 days), during which time six dogs died, including two dogs in IRIS stage 2, and four dogs in IRIS stage 3. No dogs in the control group died; no data from this group were included in the survival analysis. Causes of death included four dogs with worsening CKD (including one dog that was in IRIS stage 2 at the time of recruitment and three dogs that were in IRIS stage 3 at the time of recruitment), one dog that died suddenly, and one dog euthanased for a bleeding hepatic mass. Five dogs were either lost to follow up (one dog in IRIS stage 3) or were still alive (three dogs in IRIS stage 2 and one dog in IRIS stage 3). Median survival time in dogs with CKD was 793 days (95% confidence interval [CI] 141-NA). Results of univariate Cox proportional hazards analysis are summarized in Table 3. Risk of all-cause death increased significantly with increasing age ($P =$

0.048), serum total protein (P = 0.030), and LVPWDN (P = 0.023). All three dogs with LVPWDN values exceeding the 95% reference value at baseline died during the study. Risk of death increased significantly with decreasing bodyweight (P = 0.035), and PCV (P = 0.027).

Discussion

To the authors' knowledge, this pilot study is the first to describe echocardiographic measurements in dogs with moderate CKD in the context of proposed $CvRD_{K.}$ This condition refers specifically to presence or development of heart disease secondarily to CKD, and the current study specifically excluded dogs with evidence of primary valvular disease or cardiomyopathy. Thus, echocardiographic measurements in dogs with CKD were compared to age-matched healthy controls. Our analysis found no significant differences at time of enrolment in left heart echocardiographic measurements between groups. These findings differ from reports in human patients, wherein chronic renocardiac syndrome in patients with advanced, dialysis-dependent CKD is characterized by left ventricular hypertrophy, myocardial and arterial fibrosis, and coronary atherosclerosis leading to diastolic and systolic dysfunction, heart failure, or sudden death (Herzog et al., 2011; Tumlin et al., 2013; Granata et al., 2016). Echocardiographic changes reported in human patients with mild to moderate CKD, similar to the dogs enrolled in the present study, include increased left atrial size, increased mitral A wave velocity and tissue Doppler imaging changes consistent with diastolic dysfunction (Xhakollari et al., 2019); no differences among groups was identified for these variables in the present study. This might be due to the present study being underpowered to detect differences, especially for the tissue Doppler variables, for which data were frequently

missing, or might reflect true species differences. Additionally, in human patients, systemic hypertension leading to pressure overload is one of the main mechanisms of renocardiac syndrome (Tumlin et al., 2013), and another possible explanation for the absence of baseline echocardiographic changes in dogs with CKD in the present study was that blood pressure measurements were not significantly different from those of the control group, despite the finding that 8/11 of the dogs with CKD had elevated systolic blood pressures.

However, despite only moderate CKD, over half of the dogs with CKD, including 4/7 dogs originally diagnosed as IRIS stage 3, died during the study follow up. The median survival time in the current study (793 days) is similar to previously reported cohorts. In one previous study (Hokamp et al., 2016), dogs with serum creatinine of 1.6-2.7 mg/dL had a median survival of approximately 1.5 to 2 years.

A variety of different variables were associated with survival in dogs with CKD. An older age, lighter bodyweight, lower PCV, higher total protein, and thicker left ventricular wall thickness were associated with decreased survival. A relationship between bodyweight and survival in dogs with CKD has been previously reported, with heavier or overweight dogs surviving significantly longer than underweight dogs (Parker and Freeman, 2011). In humans with CKD, the relationship between mortality and body mass is complex, and many studies propose a U-shaped curve to describe this relationship (Mafra et al., 2008; Lu et al., 2014; Johansen and Lee, 2015). Patients that are underweight or severely overweight experience the greatest mortality, and patients that are overweight or mildly obese survive the longest, potentially due to greater caloric reserve and muscle mass, and less protein-energy

wasting and malnutrition (Mafra et al., 2008; Lu et al., 2014; Johansen and Lee, 2015).

In the present study, dogs with IRIS stage 3 CKD had significantly lower PCVs compared with dogs in the control group, consistent with the development of mild anemia secondary to CKD. Anemia is associated with progression of CKD in cats (Chakrabarti et al., 2012), poor quality of life in dogs (Bartges, 2012), and severe anemia increases risk for ventricular hypertrophy, heart failure, and reduced quality of life in human CKD patients (Horl, 2013). Although none of the dogs included in the study had evidence of clinically-significant anemia that would be expected to contribute to left ventricular hypertrophy, it is likely that the influence of PCV on survival might be related to dogs with lower PCVs having more advanced disease.

In contrast, there was no difference in serum total protein detected among groups, and so this does not appear to be related simply to disease progression. No measurements were outside the laboratory reference interval, suggesting that no dog was experiencing clinically-significant dehydration. However, it is possible that subtle dehydration secondary to renal dysfunction might be associated with a poorer prognosis – this hypothesis should be tested prospectively. An alternative explanation is that feeding of a low protein diet, which has been shown to be associated with increased survival times in dogs with CKD, might result in decreased total protein measurements.

Left ventricular hypertrophy is an important feature of CKD resulting from amongst other factors, systemic hypertension, neurohormonal activation, and

oxidative stress (Pinheiro da Silva and Vaz da Silva, 2016). Patterns of left ventricular hypertrophy reported in human CKD patients include concentric hypertrophy (typically symmetric hypertrophy or asymmetric septal hypertrophy (Ghione et al., 1985, Kooman and Leunissen, 1993)), eccentric hypertrophy and mixed patterns of hypertrophy (Cusimano et al., 2009). No evidence of concentric (either symmetric or asymmetric) or eccentric hypertrophy was found in any of the dogs enrolled in the present study, with all measurements of IVSDN, LVPWDN and LVIDDN being within the reference intervals. Nevertheless, for each 0.1 unit increase in LVPWDN, the risk of mortality increased by 27%, although no association between IVSDN and survival was demonstrated. This might suggest that even early, subtle increases in left ventricular wall thickness are an indicator of poor prognosis in dogs with CKD, even when measurements remain within the reference interval. It is noteworthy that this relationship was only demonstrated for LVPWDN, despite the typical patterns of left ventricular hypertrophy in human patients being either symmetric or involving the septum alone. Possible explanations for this difference include the study being underpowered to demonstrate an association for IVSDN, patterns of left ventricular hypertrophy in canine patients differing from those of human patients or a spurious association due to an unidentified confounding factor. This association should, therefore, be investigated further in a larger population of dogs.

One dog in the study died unexpectedly and suddenly, which could have been due to a cardiac arrhythmia. Sudden cardiac death is the most common cause of cardiac mortality in human patients with CKD, and related to autonomic imbalance,

damaged or hypertrophied myocardial substrate, and pro-arrhythmic triggers, such as acute fluid shifts and electrolyte imbalances (Poulikakos et al., 2014).

A number of markers previously-shown to be associated with outcome in dogs with CKD (e.g. GFR, serum creatinine and SDMA and UPC) were not significant in the univariate survival analysis. This is most likely to be because baseline measurements were used for the survival analysis, at which point all dogs had moderate CKD. As a result, baseline measurements were similar for all dogs included in the analysis and were therefore unlikely to discriminate between survivors and non-survivors, particularly in a small pilot study.

Our study provides new information involving renal and cardiac biomarkers with regards to differences between patient groups, as well as in relation to survival. At time of enrolment, dogs with CKD had significantly increased NT-proBNP, urinary cystatin B/ creatinine ratio, urinary clusterin/ creatinine ratio and urinary NGAL/ creatinine ratio. Increased NT-proBNP concentration might reflect increased production or decreased excretion. NT-proBNP undergoes renal clearance from the circulation, however, the relationship between plasma NT-proBNP and GFR is controversial. The observation that plasma NT-proBNP is higher in azotemic dogs compared with non-azotemic dogs has long been assumed to represent accumulation due to decreased GFR (Raffan et al., 2009), however, a previous study showed that GFR was not an independent predictor of plasma NT-proBNP concentration (Pelander et al., 2017). In the current study plasma NT-proBNP was only moderately correlated with GFR, suggesting other factors probably contribute to increased plasma NT-proBNP in dogs with CKD. An increase in NT-proBNP might also reflect an

increase in production, for instance, in response to increased cardiac filling pressures or neurohormonal activation. In addition to NT-proBNP, three separate markers of active kidney injury, urinary cystatin B, clusterin and NGAL, were increased in dogs with CKD. Cystatin B is a protein inhibitor of cysteine proteases, whereas NGAL is a protein that binds iron-containing ligands (Steinbach et al., 2014). Serum NGAL is increased in dogs with congestive heart failure, consistent with $CvRD_H$ (Jung et al., 2018). Clusterin is a di-sulfide-linked glycoprotein that has protective, anti-apoptotic, and anti-fibrotic properties (Jung et al., 2012), and is upregulated in response to tubular epithelial injury in dogs (Garcia-Martinez et al., 2012; Yerramilli et al., 2016). All three are found in renal tubular cells, and detection in the blood or urine is probably associated with active tubular injury or necrosis that has caused rupture of renal tubular epithelial cells. Increased concentrations of protective proteins such as clusterin and NT-proBNP might be a marker of disease severity, signal inadequate biological efficacy, or altered catabolism or excretion.

The current pilot study has a number of limitations. Standard echocardiography was performed, and detection of subtle differences in cardiac structure or function might require more sensitive ultrasound techniques, or alternative techniques such as MRI or cardiac catheterization. Dogs with advanced CKD, or those with uncontrolled systemic hypertension, were not examined. The study population was small, which prevents the ability to perform multivariable survival analysis. Variables identified as being associated with survival should therefore be confirmed in larger patient groups.

Conclusions

In this study, dogs with moderate CKD demonstrated increased markers of cardiac stress and kidney injury compared to age-matched controls. While there was no significant difference in echocardiographic measurements between CKD and healthy controls, LVPWDN was associated with worse survival in dogs with CKD. These data help provide insight into the proposed phenomenon of $CvRD_K$, provide information regarding baseline heart measurements in dogs with moderate CKD, and are amongst the first to investigate an association between echocardiographic indices and mortality in dogs with CKD.

Conflict of Interest Statement

Drs. Hezzell and Oyama have previously received research funding, reimbursement of travel expenses, honoraria, consulting fees, and programmatic support from Ceva Santé Animale and IDEXX Laboratories. Drs. Buch, Farace, Quinn and Yerramilli are employed by IDEXX Laboratories. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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References

Acierno, M.J., Brown, S., Coleman, A.E., Jepson, R.E., Papich, M., Stepien, R.L., Syme, H.M., 2018. ACVIM consensus statement: Guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats. Journal of Veterinary Internal Medicine 32, 1803-1822.

Alhaj, E., Alhaj, N., Rahman, I., Niazi, T.O., Berkowitz, R., Klapholz, M., 2013. Uremic cardiomyopathy: an underdiagnosed disease. Congestive Heart Failure 19, e40-45.

Bartges, J.W., 2012. Chronic kidney disease in dogs and cats. Veterinary Clinics of North America Small Animal Practice 42, 669-692.

Bernardi D, Bernini L, Cini G, Ghione S, Bonechi I., 1985. Asymmetric septal hypertrophy and sympathetic overactivity in normotensive hemodialyzed patients. American Heart Journal 109, 539-545.

Cahill, R.J., Pigeon, K., Strong-Townsend, M.I., Drexel, J.P., Clark, G.H., Buch, J.S., 2015. Analytical validation of a second-generation immunoassay for the quantification of N-terminal pro-B-type natriuretic peptide in canine blood. Journal of Veterinary Diagnostic Investigation 27, 61-67.

Chakrabarti, S., Syme, H.M., Elliott, J., 2012. Clinicopathological variables predicting progression of azotemia in cats with chronic kidney disease. Journal of Veterinary Internal Medicine 26, 275-281.

Clementi, A., Virzi, G.M., Goh, C.Y., Cruz, D.N., Granata, A., Vescovo, G., Ronco, C., 2013. Cardiorenal syndrome type 4: a review. Cardiorenal Medicine 3, 63-70.

Cornell, C.C., Kittleson, M.D., Della Torre, P., Haggstrom, J., Lombard, C.W., Pedersen, H.D., Vollmar, A., Wey, A., 2004. Allometric scaling of M-mode cardiac measurements in normal adult dogs. Journal of Veterinary Internal Medicine 18, 311-321.

Dickson D, Shave R, Rishniw M, Rishniw M, Patteson M., 2017. Echocardiographic assessments of longitudinal left ventricular function in healthy English Springer spaniels. Journal of Veterinary Cardiology 19, 339-350.

Dukes-McEwan, J., Borgarelli, M., Tidholm, A., Vollmar, A.C., Haggstrom, J., 2003. Proposed guidelines for the diagnosis of canine idiopathic dilated cardiomyopathy. Journal of Veterinary Cardiology 5, 7-19.

Garcia-Martinez, J.D., Tvarijonaviciute, A., Ceron, J.J., Caldin, M., Martinez-Subiela, S., 2012. Urinary clusterin as a renal marker in dogs. Journal of Veterinary Diagnostic Investigation 24, 301-306.

Granata, A., Clementi, A., Virzi, G.M., Brocca, A., de Cal, M., Scarfia, V.R., Zanoli, L., Ronco, C., Corrao, S., Malatino, L., 2016. Cardiorenal syndrome type 4:

From chronic kidney disease to cardiovascular impairment. European Journal of Internal Medicine 30, 1-6.

Haggstrom, J., Hansson, K., Karlberg, B.E., Kvart, C., Olsson, K., 1994. Plasma concentration of atrial natriuretic peptide in relation to severity of mitral regurgitation in Cavalier King Charles Spaniels. American Journal of Veterinary Research 55, 698-703.

Hansson, K., Haggstrom, J., Kvart, C., Lord, P., 2002. Left atrial to aortic root indices using two-dimensional and M-mode echocardiography in cavalier King Charles spaniels with and without left atrial enlargement. Veterinary Radiology and Ultrasound 43, 568-575.

Heiene, R., Eliassen, K.A., Risoen, U., Neal, L.A., Cowgill, L.D., 2010. Glomerular filtration rate in dogs as estimated via plasma clearance of inulin and iohexol and use of limited-sample methods. American Journal of Veterinary Research 71, 1100-1107.

Herzog, C.A., Asinger, R.W., Berger, A.K., Charytan, D.M., Diez, J., Hart, R.G., Eckardt, K.U., Kasiske, B.L., McCullough, P.A., Passman, R.S., DeLoach, S.S., Pun, P.H., Ritz, E., 2011. Cardiovascular disease in chronic kidney disease. A clinical update from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney International 80, 572-586.

Hokamp, J.A., Cianciolo, R.E., Boggess, M., Lees, G.E., Benali, S.L., Kovarsky, M., Nabity, M.B., 2016. Correlation of Urine and Serum Biomarkers with Renal Damage and Survival in Dogs with Naturally Occurring Proteinuric Chronic Kidney Disease. Journal of Veterinary Internal Medicine 30, 591-601.

Horl, W.H., 2013. Anaemia management and mortality risk in chronic kidney disease. Nature Reviews Nephrology 9, 291-301.

Johansen, K.L., Lee, C., 2015. Body composition in chronic kidney disease. Current Opinion in Nephrology and Hypertension 24, 268-275.

Jung, G.S., Kim, M.K., Jung, Y.A., Kim, H.S., Park, I.S., Min, B.H., Lee, K.U., Kim, J.G., Park, K.G., Lee, I.K., 2012. Clusterin attenuates the development of renal fibrosis. Journal of the American Society of Nephrology 23, 73-85.

Jung, H.B., Kang, M.H., Park, H.M., 2018. Evaluation of serum neutrophil gelatinase-associated lipocalin as a novel biomarker of cardiorenal syndrome in dogs. Journal of Veterinary Diagnostic Investigation 30, 386-391.

Keller, S.P., Kovacevic, A., Howard, J., Schweighauser, A., Francey, T., 2016. Evidence of cardiac injury and arrhythmias in dogs with acute kidney injury. Journal of Small Animal Practice 57, 402-408.

Kingma, J.G., Simard, D., Rouleau, J.R., Drolet, B., Simard, C., 2017. The Physiopathology of Cardiorenal Syndrome: A Review of the Potential

Contributions of Inflammation. Journal of Cardiovascular Development and Disease 4, e21.

Kooman J.P., Leunissen K.M., 1993. Cardiovascular aspects in renal disease. Current Opinion in Nephrology and Hypertension 2, 791-797.

Lu, J.L., Kalantar-Zadeh, K., Ma, J.Z., Quarles, L.D., Kovesdy, C.P., 2014. Association of body mass index with outcomes in patients with CKD. Journal of the American Society of Nephrology 25, 2088-2096.

Mafra, D., Guebre-Egziabher, F., Fouque, D., 2008. Body mass index, muscle and fat in chronic kidney disease: questions about survival. Nephrology Dialysis Transplantation 23, 2461-2466.

Martinelli, E., Locatelli, C., Bassis, S., Crosara, S., Paltrinieri, S., Scarpa, P., Spalla, I., Zanaboni, A.M., Quintavalla, C., Brambilla, P., 2016. Preliminary Investigation of Cardiovascular-Renal Disorders in Dogs with Chronic Mitral Valve Disease. Journal of Veterinary Internal Medicine 30, 1612-1618.

Muntner, P., He, J., Hamm, L., Loria, C., Whelton, P.K., 2002. Renal insufficiency and subsequent death resulting from cardiovascular disease in the United States. Journal of the American Society of Nephrology 13, 745-753.

Nabity, M.B., Lees, G.E., Boggess, M.M., Yerramilli, M., Obare, E., Yerramilli, M., Rakitin, A., Aguiar, J., Relford, R., 2015. Symmetric Dimethylarginine Assay Validation, Stability, and Evaluation as a Marker for the Early Detection of Chronic Kidney Disease in Dogs. Journal of Veterinary Internal Medicine 29, 1036-1044.

Nardi E, Palermo A, Mule G, Cusimano P, Cottone S, Cerasola G., 2009. Left ventricular hypertrophy and geometry in hypertensive patients with chronic kidney disease. Journal of Hypertension 27, 633-641.

Parker, V.J., Freeman, L.M., 2011. Association between body condition and survival in dogs with acquired chronic kidney disease. Journal of Veterinary Internal Medicine 25, 1306-1311.

Pelander, L., Haggstrom, J., Ley, C.J., Ljungvall, I., 2017. Cardiac Troponin I and Amino-Terminal Pro B-Type Natriuretic Peptide in Dogs With Stable Chronic Kidney Disease. Journal of Veterinary Internal Medicine 31, 805-813.

Pinheiro da Silva, A.L., Vaz da Silva, M.J., 2016. Type 4 cardiorenal syndrome. Revista Portuguesa de Cardiologia 35, 601-616.

Pouchelon, J.L., Atkins, C.E., Bussadori, C., Oyama, M.A., Vaden, S.L., Bonagura, J.D., Chetboul, V., Cowgill, L.D., Elliot, J., Francey, T., Grauer, G.F., Fuentes, V.L., Moise, N.S., Polzin, D.J., Van Dongen, A.M., Van Israel, N., 2015. Cardiovascular-renal axis disorders in the domestic dog and cat: a veterinary consensus statement. Journal of Small Animal Practice 56, 537-552.

Poulikakos, D., Banerjee, D., Malik, M., 2014. Risk of sudden cardiac death in chronic kidney disease. Journal of Cardiovascular Electrophysiology 25, 222-231.

Raffan, E., Loureiro, J., Dukes-McEwan, J., Fonfara, S., James, R., Swift, S., Bexfield, N., Herrtage, M.E., Archer, J., 2009. The cardiac biomarker NT-proBNP is increased in dogs with azotemia. Journal of Veterinary Internal Medicine 23, 1184-1189.

Rangaswami, J., Bhalla, V., Blair, J.E.A., Chang, T.I., Costa, S., Lentine, K.L., Lerma, E.V., Mezue, K., Molitch, M., Mullens, W., Ronco, C., Tang, W.H.W., McCullough, P.A., 2019. Cardiorenal Syndrome: Classification, Pathophysiology, Diagnosis, and Treatment Strategies: A Scientific Statement From the American Heart Association. Circulation 139, e840-e878.

Ronco, C., Di Lullo, L., 2014. Cardiorenal syndrome. Heart Failure Clinics 10, 251-280.

Ronco, C., Haapio, M., House, A.A., Anavekar, N., Bellomo, R., 2008. Cardiorenal syndrome. Journal of the American College of Cardiology 52, 1527-1539.

Steinbach, S., Weis, J., Schweighauser, A., Francey, T., Neiger, R., 2014. Plasma and urine neutrophil gelatinase-associated lipocalin (NGAL) in dogs with acute kidney injury or chronic kidney disease. Journal of Veterinary Internal Medicine 28, 264-269.

Tumlin, J.A., Costanzo, M.R., Chawla, L.S., Herzog, C.A., Kellum, J.A., McCullough, P.A., Ronco, C., 2013. Cardiorenal syndrome type 4: insights on clinical presentation and pathophysiology from the eleventh consensus conference of the Acute Dialysis Quality Initiative (ADQI). Contributions to Nephrology 182, 158-173.

Xhakollari L, Leosdottir M, Magnusson M, Holzmann M.J., Nilsson P.M., Christensson A., 2019. Echocardiographic Findings in Patients with Mild to Moderate Chronic Kidney Disease without Symptomatic Heart Failure: A Population-Based Study. Cardiorenal Medicine 9, 284-296.

Yerramilli, M., Farace, G., Quinn, J., Yerramilli, M., 2016. Kidney Disease and the Nexus of Chronic Kidney Disease and Acute Kidney Injury: The Role of Novel Biomarkers as Early and Accurate Diagnostics. Veterinary Clinics of North America Small Animal Practice 46, 961-993.

Table 1

Summary of dog characteristics, serum biochemistry, and urinalysis at enrolment. Values are reported as median and range.

	Reference interval	**Control (n = 12)**	**IRIS Stage 2 (n = 5)**	**IRIS Stage 3 (n = 6, unless otherwise specified)**	***P* (overall groupwise comparison)**	***Post hoc* groupwise comparisons where *P*<0.05**
Age (years)	N/A	9.0 (2.6 – 14.1)	8.0 (3.3 – 15.6)	9.6 (1.2 – 12.5)	0.922	N/A
Bodyweight (kg)	N/A	21.9 (11.6 – 30.9)	33.3 (10.0 – 38.5)	27.4 (11.2 – 42.4)	0.433	N/A
Heart rate (bpm)	60-180	123 (90 – 150)	108 (100 – 120)	112 (80 – 130)	0.078	N/A
Systolic blood pressure (mmHg)	110-160	140.5 (110.0 – 178.0)	170.0 (128.0 – 184.0)	173.0 (130.0 – 210.0)	0.090	N/A
Packed cell volume (%)	41-58	49.0 (42.0 – 54.0)	49.0 (38.0 – 53.0)	38.0 (33.0 – 44.0) (n = 5)	0.013 [a]	Control vs. IRIS 3, *P*=0.017
Serum total protein (g/dL)	5.0-8.0	6.5 (6.0 – 7.8)	7.0 (6.2 – 7.9)	6.8 (6.0 – 8.0)	0.363	N/A
BUN (mg/dL)	5-30	14 (9 – 22)	20 (16 – 37)	27 (18 – 81)	0.002 [a]	Control vs. IRIS 3, *P*=0.003
Creatinine (mg/dL)	0.7-1.8	1.0 (0.8 – 1.5)	1.7 (1.4 – 1.9)	2.4 (2.3 – 3.1)	0.0001 [a]	Control vs. IRIS 3, *P*=0.0001
Phosphorus (mg/dL)	2.8-6.1	3.9 (3.0 – 5.3)	4.0 (3.3 – 4.8)	4.6 (3.3 – 6.8)	0.392	N/A
Na^+ (mmol/L)	140-150	146.5 (142.0 – 151.0)	143.0 (139.0 – 147.0)	142.5 (139.0 – 145.0)	0.011 [a]	Control vs. IRIS 3, *P*=0.012
K^+ (mmol/L)	4.0-5.2	4.6 (4.0 – 5.0)	4.7 (4.2 – 5.2)	4.7 (4.4 – 5.9)	0.353	N/A
USG	>1.025	1.036 (1.010 – 1.061)	1.010 (1.008 – 1.018)	1.010 (1.002 – 1.016)	0.001 [a]	Control vs. IRIS 2, *P*=0.025; Control vs. IRIS 3, *P*=0.003
UPC	<0.50	0.02 (0.01 – 0.15)	0.09 (0.08 – 1.82)	0.60 (0.10 – 5.33)	0.001 [a]	Control vs. IRIS 2; *P*=0.048, Control vs. IRIS 3; *P*=0.002

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4 BUN; blood urea nitrogen, Na^+; sodium, K^+; potassium, USG; urinary specific gravity, UPC; urinary protein to creatinine ratio.

5 [a] $P<0.05$

Table 2

Summary of GFR, plasma biomarker measurements, and basic echocardiographic data at enrollment. Values are reported as median and range.

	Reference interval	**Control (n = 12, unless otherwise specified)**	**IRIS Stage 2 (n = 5)**	**IRIS Stage 3 (n = 6, unless otherwise specified)**	***P*** **(overall groupwise comparison)**	**Post hoc groupwise comparisons where *P*<0.05**
GFR (mL/ min/ kg)	Expected mean = 5.48	2.33 (1.71 – 4.33) (n=11)	1.20 (1.05 – 1.49)	0.76 (0.55 - 1.04)	<0.0001 [a]	Control vs. IRIS 2, *P*=0.050; Control vs. IRIS 3, *P*<0.001
SDMA (mg/dL)	<14	11.0 (8.0 – 14.0)	16.0 (14.0 – 25.0)	24.0 (16.0 – 30.0)	<0.0001 [a]	Control vs. IRIS 2, *P*=0.028; Control vs. IRIS 3, *P*<0.001; IRIS 2 vs. IRIS 3, *P*=0.003
Plasma NT-proBNP (pmol/L)	<900	438 (250 – 1433)	604 (306 – 1567)	1788 (1295 – 2881) (n=5)	0.001 [a]	Control vs. IRIS 3, *P*=0.003
Plasma cTnI (ng/mL)	<0.08	0.02 (0.00 – 0.08)	0.01 (0.00 – 0.12)	0.04 (0.00 – 0.05)	0.830	N/A
Plasma cystatin B (ng/mL)	N/A	199.0 (109.0 – 325.0)	239.0 (174.0 – 311.0)	218.5 (150.0 – 260.0)	0.599	N/A
Urinary cystatin B/ creatinine ratio (ng/mg)	N/A	0.006 (0.00 – 1.95)	7.03 (0.00 – 14.87)	5.70 (0.59 – 13.46) (n=4)	0.005 [a]	Control vs. IRIS 2, *P*=0.077, Control vs. IRIS 3, *P*=0.037
Urinary clusterin/ creatinine ratio (ng/mg)	N/A	0.26 (0.07 – 8.63)	2.05 (0.47 – 6.69)	4.05 (2.94 – 13.74) (n = 4)	0.019 [a]	Control vs. IRIS 3, *P*=0.036
Urinary NGAL/ creatinine ratio (ng/mg)	N/A	0.00 (0.00 – 0.06)	0.28 (0.00 – 0.47)	0.57 (0.05 – 0.81) (n = 4)	<0.001 [a]	Control vs. IRIS 2, *P*=0.072, Control vs. IRIS 3, *P*=0.005

IVSDN (cm/[kg$^{.241}$])	0.29 – 0.59	0.44 (0.37 - 0.50)	0.50 (0.38 - 0.54)	0.50 (0.33 - 0.57)	0.125	N/A
LVPWDN (cm/[kg$^{.232}$])	0.29 - 0.60	0.44 (0.36 - 0.59)	0.51 (0.42 - 0.59)	0.50 (0.43 - 0.59)	0.284	N/A
LVIDDN (cm/[kg$^{.294}$])	1.27 - 1.85	1.50 (1.30 – 1.60)	1.30 (1.20 – 1.50)	1.60 (1.30 – 1.60)	0.096	N/A
LVIDSN (cm/[kg$^{.315}$])	0.71 – 1.26	0.91 (0.79 – 1.10)	0.79 (0.63 - 0.96)	0.87 (0.78 – 1.00)	0.257	N/A
Fractional shortening (%)	20 - 55	32.7 (20.2 – 39.4)	35.9 (25.0 – 43.0)	35.3 (29.6 – 67.8)	0.305	N/A
LA:Ao	<1.6	1.20 (0.91 – 1.40)	1.20 (0.95 – 1.30)	1.15 (0.93 – 1.40)	0.946	N/A
Mitral E wave velocity (m/s)	0.48 – 1.08	0.67 (0.40 - 0.95)	0.67 (0.48 - 0.86)	0.83 (0.58 – 1.14)	0.106	N/A
Mitral A wave velocity (m/s)	0.30 - 0.80	0.63 (0.44 - 0.94)	0.66 (0.56 - 0.88)	0.71 (0.57 - 1.01)	0.352	N/A
Mitral EA ratio	1 - 2	1.06 (0.65-1.63)	0.80 (0.72-1.29)	1.03 (0.66-1.64)	0.768	N/A
Mitral E'wave velocity (cm/s)	3.7 - 17.1	8 (14 – 12)	6 (5 – 7) (n = 3)	6 (5 – 8) (n = 3)	0.153	N/A
Mitral E/E' ratio (m/cm)	10.9 ± 3.3	8.0 (4.0 – 17.0)	11.0 (7.0 – 15.0) (n = 3)	15.0 (10.0 – 17.0) (n = 3)	0.090	N/A

GFR; glomerular filtration rate, SDMA; symmetric dimethylarginine, NGAL; neutrophil gelatinase-associated lipocalin, NT-proBNP; N-terminal pro-B-type natriuretic peptide, cTnI; cardiac troponin I, IVSDN; interventricular septal wall thickness in diastole, normalized for bodyweight, LVPWDN; left ventricular posterior wall thickness in diastole, normalized for bodyweight, LVIDDN; left ventricular internal dimension in diastole, normalized for bodyweight, LVIDSN; left ventricular internal dimension in systole, normalized for bodyweight, LA:Ao; left atrial to aortic root ratio; N/A, not available.

[a] $P<0.05$

Table 3

Univariate Cox proportional hazards analysis of factors associated with all-cause mortality at enrolment into the study.

	Hazard ratio (HR)	95% confidence interval for HR	P
Age (years)	1.330	1.002 - 1.764	0.048 [a]
Bodyweight (kg)	0.891	0.801 - 0.992	0.035 [a]
Heart rate (bpm)	1.083	0.988 - 1.187	0.090
Systolic blood pressure (mmHg)	1.013	0.976 - 1.051	0.509
Packed cell volume (%) (n=10)	0.745	0.575 - 0.967	0.027 [a]
Serum total protein (.1 g/dL)	1.458	1.037 - 2.049	0.030 [a]
GFR (mL/min/kg)	0.248	0.013 - 4.771	0.356
UPC (mg/g)	1.520	0.741 - 3.121	0.254
BUN (mg/dL)	1.154	0.983 - 1.354	0.079
Creatinine (mg/dL)	2.801	0.615 - 12.750	0.183
Phosphorus (mg/dL)	1.848	0.909 - 3.759	0.090
SDMA (mg/dL)	1.036	0.900 - 1.192	0.626
NT-proBNP (100 pmol/L) (n=10)	1.020	0.916 - 1.136	0.717
cTnI x (.01 ng/mL)	0.957	0.705 -1 .298	0.777
Plasma cystatin B (10ng/mL) (n=10)	1.030	0.857 - 1.238	0.751
Urinary cystatin B/ creatinine ratio (1ng/mg) (n = 9)	1.167	0.954 - 1.427	0.133
Urinary clusterin/ creatinine ratio (1ng/mg) (n = 9)	1.526	0.948 - 2.454	0.082
Urinary NGAL/ creatinine ratio (1ng/mg) (n = 9)	27.353	0.637 - 1175.308	0.085
IVSDN (0.1 unit)	1.122	0.954 - 1.319	0.163
LVPWDN (0.1 unit)	1.273	1.034 - 1.567	0.023 [a]

LVIDDN (0.1 unit)	0.997	0.939 - 1.058	0.920
LVIDSN (0.1 unit)	0.965	0.906 - 1.029	0.275
LA:Ao (0.1 unit)	0.773	0.467 - 1.279	0.316
Mitral E wave velocity (0.1 m/s)	0.782	0.458 - 1.334	0.367
Mitral EA ratio (0.1 units)	0.793	0.580 - 1.084	0.146
Mitral E'wave velocity (cm/s)	0.965	0.371 - 2.513	0.942
Mitral E/E' ratio (m/cm)	0.488	$0.000 – 2.670E^{13}$	0.965

BUN; blood urea nitrogen, Na^+; sodium, K^+; potassium, USG; urinary specific gravity, UPC; urinary protein to creatinine ratio, GFR; glomerular filtration rate, SDMA; symmetric dimethylarginine, NGAL; neutrophil gelatinase-associated lipocalin, NT-proBNP; N-terminal pro-B-type natriuretic peptide, cTnI; cardiac troponin I, IVSDN; interventricular septal wall thickness in diastole, normalized for bodyweight, LVPWDN; left ventricular posterior wall thickness in diastole, normalized for bodyweight, LVIDDN; left ventricular internal dimension in diastole, normalized for bodyweight, LVIDSN; left ventricular internal dimension in systole, normalized for bodyweight, LA:Ao; left atrial to aortic root ratio.

[a] $P<0.05$